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# Mutant breeding of *Aspergillus niger* irradiated by $^{12}C^{6+}$ for hyper citric acid*

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In this study, strains of *Aspergillus niger* 4# for hyper citric acid were irradiated to different doses by 80 MeV/u $^{12}C^{6+}$ ion beams. Seven mutant strains showed marked citric acid over-production records and faster productivity than initial *Aspergillus niger* 4# by shaking flash fermentation. The maximum product yield was 132.8 g L$^{-1}$( the H4002 strain) being a 8.8% increase to the initial strain. The scale-up experiment was carried out in a 100 L bioreactor. The mutant H4002 can accumulate 187 g L$^{-1}$ product yield of citric acid from starch liquefying supernatant. The productivity of citric acid was 2.75 g L$^{-1}$ h$^{-1}$. So, the mutant H4002 possesses rapid sugar katabolism for producing citric acid. Meanwhile, the pellet morphology kept compact and round during the whole submerged fermentation, which was suited to produce citric acid. The results indicate that mutant H4002 has potential ability to produce citric acid rapidly.

Keywords: $^{12}C^{6+}$ ion, Rapid fermentation, Productivity, Pellets morphology

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## I. INTRODUCTION

Citric acid fermentation is one of the largest biotechnological industries [1] and *Aspergillus niger* is of a good potential to produce citric acid. The annual citric acid production through fermentation is estimated at $7.0 \times 10^5$ ton [2] and the industrial demand of citric acid is in steady increase. In 2008, the best citric acid production level of Chinese producers is 146.9 g L$^{-1}$ and the best fermentation index is 2.7 g L$^{-1}$ h$^{-1}$ [3], both being much lower than the advanced levels in the world. Therefore, it is vital to enhance the citric acid production capacity of *Aspergillus niger*. Aimed at obtaining high product yield in production scale, a possible strategy is to improve microbial production strain.

Strains with superior characters, such as enhanced citric acid production and high fermentation index have been selected after subjecting the genetic material to physical or chemical mutagenic agents [4, 5]. Heavy ion-induced mutation is a unique method of physical mutation. With high LET (linear energy transfer) and RBE (relative biological effectiveness) [6], it can induce single- or double-strand DNA breaks with low reparability in bombarded tissue [7] and mighty potency to improve the mutation rate and broaden the mutation spectrum [8]. Progresses have been made at Institute of Modern Physics, Chinese Academy of Science (IMP, CAS) in heavy ion mutation breeding of Gentamicin, alcohol-resistant yeast and Streptomyces Avermitilis [9, 10].

In this paper, we report the selection of mutants of *A.niger* for hyper production of citric acid irradiated by $^{12}C^{6+}$ beams form Heavy Ion Research Facility of Lanzhou (HIRFL). The bioreactor is applied to reveal fermentation characters of the best mutant strain for citric acid production and the pellet morphology of different fermentation cycle is also analyzed.

## II. MATERIALS AND METHODS

### A. Microorganism

Initial *Aspergillus niger* was provided by biophysics lab of IMP, CAS. It was maintained on potato dextrose agar slants and stored at 4 °C in a refrigerator and Spores of *Aspergillus niger* were inoculated in the bran medium (20% potato, 2% saccharose, 2% agar) for 6 d.

### B. Irradiation

The conidial suspension of 6 d old slant culture of *Aspergillus niger* 4# in saline water was transferred to irradiation dish. The colony-forming units/mL (CFU/mL) were maintained at $1 \times 10^6$ cells/mL. Seven groups of initial *Aspergillus niger* strains were prepared and irradiated to 0 Gy, 10 Gy, 20 Gy, 30 Gy, 40 Gy, 50 Gy and 60 Gy. The $^{12}C^{6+}$ ion beams of 80 MeV/u have an LET of 40 keV μm$^{-1}$.

### C. Screening of mutant strains for hyper citric acid

Colony suspensions irradiated to different doses were diluted to $10^{-5}$ gradient. Then, 0.1 mL diluted colony suspensions was to daubed on the solid plate medium. The strains with high ratio between acid spot diameter and lawn diameter was selected for shaking flash fermentation. All the fermentation mediums were composed of 16% corn starch liquefying and 0.43% mixed soybean cake powder. Initial pH was adjusted to 6.1–6.4. Compositions of the solid plate

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TABLE 1. Citric acid production by 80 Mev/u $^{12}C^{6+}$ ion beam treated mutant strains of *Aspergillus niger* following growth on corn starch liquefying (16% w/v, carbohydrates) in 250 ml Erlenmeyer flasks. Conversion rate: g citric acid produced/g substrate consumed. Productivity: citric acid concentration (g $L^{-1}$) / fermentation cycle (h)

| Mutant strains [a] | Citric acid concentration (g $L^{-1}$) | Conversion rate (%) (w/w) | Productivity (g $L^{-1}$ $h^{-1}$) |
|---|---|---|---|
| Initial strain 4# | 122.0±1.2 | 76 | 1.42 |
| H3002 (30 Gy) | 129.1±4.8 | 80 | 1.50 |
| H4001 (40 Gy) | 132.3±2.4 | 82 | 1.53 |
| H4002 (40 Gy) | 132.8±0.4 | 83 | 1.54 |
| H5001 (50 Gy) | 124.9±0.9 | 78 | 1.45 |
| H6003 (60 Gy) | 124.0±0.5 | 77 | 1.44 |
| H6005 (60 Gy) | 127.0±2.0 | 79 | 1.47 |
| H6007 (60 Gy) | 127.8±1.5 | 79 | 1.48 |

[a] Cultural conditions: temperature 37±0.4 °C, initial sugar concentration 160 g $L^{-1}$, initial pH 6.1–6.4, shaking speed 300 r $min^{-1}$, fermentation cycle 86 h. Each value is an average of three replicates.

medium were 20% potato juice, 2% sucrose, 2% agar, and 0.01% bromcresol green. The liquid mediums were sterilized at 115 °C for 30 min.

### D. Bioreactor

Fermentation was carried out in a 100 L stirred tank reactor. The fermentation mediums were prepared as follows: water to corn starch ratio 3:1, and adding 25 mL thermostable $\alpha$-amylase into 100 L bioreactor at 95 °C for 30 min. Liquefying supernatant was obtained and diluted with tap water to desired sugar concentration, and then source of nitrogen was added. The liquid mediums were sterilized at 115 °C for 30 min.

### E. Analysis

The concentration of citric acid in culture filtrate was measured by titration with 0.1429 N sodium hydroxide using 0.5% phenolphthalein as an indicator and the purity of citric acid in the fermentation was implemented by high performance liquid chromatography (HPLC). Total and residual sugars were hydrolyzed using 6 N hydrochloric acid at boiling temperature for 10 min into glucose and fructose and analyzed by a fehling reagent method.

## III. RESULTS AND DISCUSSION

### A. Mutagenesis and selecting for citric acid hyper production mutants

In the present study, a screened culture of initial *Aspergillus niger* 4# and seven mutants were examined for producing citric acid from 160 g $L^{-1}$(w/v) carbohydrates using corn starch liquefying in 250 mL Erlenmeyer flasks. The 60 Gy groups had the highest fatality rate and positive mutant rate [12]. H3002, H4001, H4002, H5001, H6003, H6005 and H6007 strains showed marked citric acid over-production records and faster productivity (Table 1), with the 40 Gy H4002 being the best.

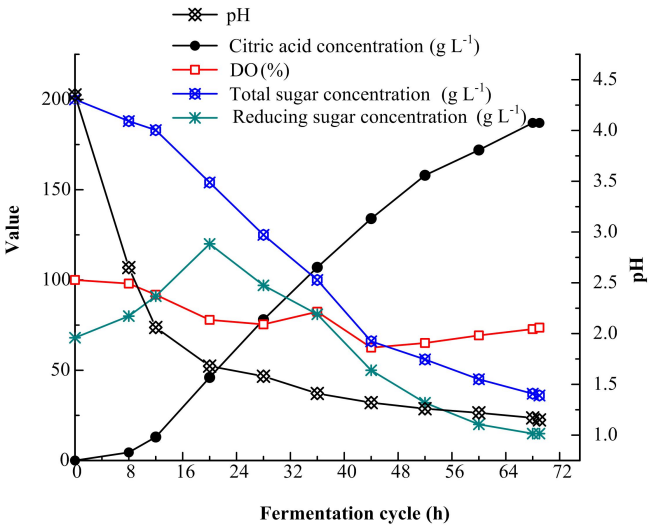


Fig. 1. (Color online) Different fermentation parameters of 200 g $L^{-1}$ sugar concentration by mutant H4002 with fermentation condition of rotate speed 400 r $min^{-1}$, aeration rate 18 L $min^{-1}$, certain tankpressure, temperature 37 °C.

### B. Analysis of fermentation characters and pellet morphology by H4002 strain in the bioreactor

Citric acid overflow requires a unique combination of several unusual nutrient conditions (i.e. high sugar concentration, $H^+$, and dissolved oxygen). In 2008, the best citric acid production level of citric acid companies in China was 146.9 g $L^{-1}$ and the best fermentation index was 2.7 g $L^{-1}$ $h^{-1}$ [3]. The H4002 strain obtained in our laboratory, however, can convert high starch liquefying sugar to citric acid more efficiently. The results (Fig. 1 show that citric acid concentration by the mutant H4002 (187 g $L^{-1}$) is much higher than initial strain (136 g $L^{-1}$), and increase of 37.5%. Productivity by mutant H4002 (2.75 g $L^{-1}$ $h^{-1}$) is 1.20 times higher than the initial strain (2.3 g $L^{-1}$ $h^{-1}$) and higher than the result in Ref. [13]. To mutant H4002 strain, it can convert sugar to citric acid with the average productivity of 2.12 g $L^{-1}$ $h^{-1}$ in the later phase of submerged fermen-

tation (44∼69 h). Furthermore, pH in the broth is very low (pH⩽1.32). For this reason, it can be deduced that H4002 strain can be far more resistant to high sugar concentration and citric acid concentration than current *Aspergillus niger* strains.

A possible explanation is that the H4002 strain has strong ability to secrete high-active glycolytic enzymes, which enhance polysaccharide catabolism. It is accepted that high activities of glycolytic enzymes can increase the rate of citric acid accumulation [14]. Ambient pH strongly influences the synthesis of secreted enzymes, permeases and metabolites by a wide range of micro-organisms [15]. The low pH (pH⩽1.32) can induce some genes over-encoding in H4002 strain [16] which make the strain itself adjust to the changing of environment conditions. Of course, this explanation needs experiment verification.

The dissolved oxygen (DO) curve show that DO value is higher than 62.5%, which is necessary to normal citric acid fermentation in the whole process. Because high DO can induce strain aging and low DO can decrease productivity [17], the optimized fermentation condition (aeration rate 18 L min$^{-1}$, rotatespeed 400 r min$^{-1}$) can meet the production need of citric acid by H4002 strain.

High performance liquid chromatography (HPLC) was applied to determine the citric acid concentration in fermentation broth. A linear regression between sample concentration ($x$) and corresponding peak area ($y$) was performed, with $y = 1.93 \times 10^9 x + 46895$, and the correlation coefficient of $R^2 = 0.9998$. The final citric acid concentration of 187 g L$^{-1}$, and the heteroacid peaks in chromatogram (Fig. 2) of fermentation broth (69 h), are encouraging. By the ratio of citric acid monohydrate (indirect measured by HPLC) to total acid (direct measured by acid-base titration), the purity of citric acid in fermentation broth is 98.4%.

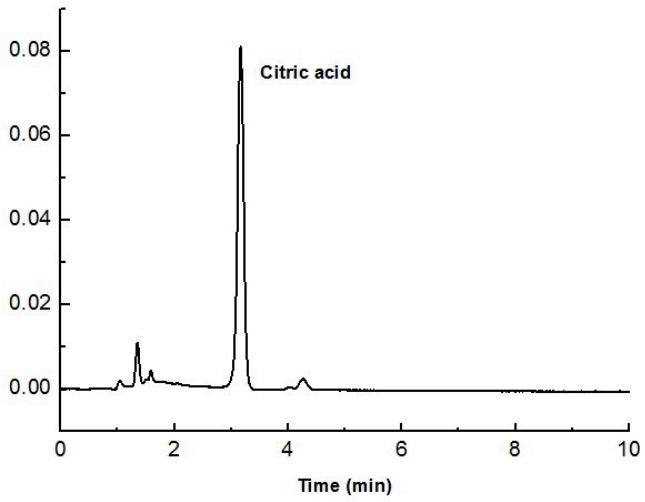


Fig. 2. The chromatogram of fermentation broth (69 h).

Figure 3 shows the pellet morphology of H4002 strain in liquid medium at 24 h, 48 h and 60 h. Most of the pellets (Fig. 3 cp) by H4002 strain are compact and round with short hyphae around pellets surface (Fig. 3 hp). However, comparing to the pellets morphology of H4002 strain in the liquid medium, the pellets morphology of 4# strain are relatively loose with relatively long hyphae around pellets surface. It is accepted that mycelial morphology of *Aspergillus niger* in citric acid fermentations is a prerequisite for industrial application. The shorter and highly branched hyphae which are the only parameter that can be helpful for the formation of pellets benefitted enhancing of citric acid [18]. However, as the citric acid concentration increases and the fermentative sugar decreases, physical property of fermentation broth changes [19], causing serious influence on pellets morphology, which can no longer keep itself compact and round. The rate of produced citric acid becomes slower.

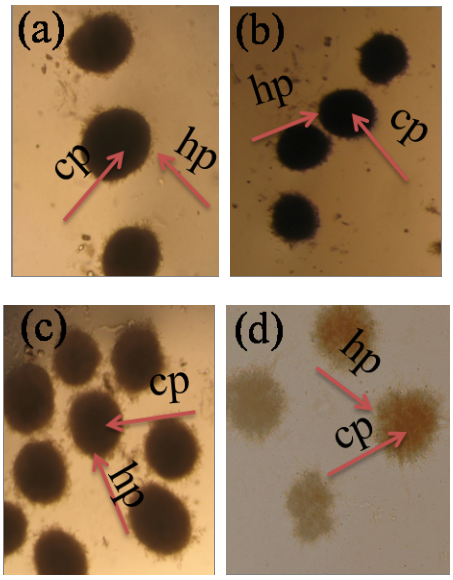


Fig. 3. (Color online) The pellet morphology of H4002strain in liquid medium for 24 h (a), 48 h (b), 60 h (c). The pellet morphology of 4# strain in liquid medium for 24 h (d). All photos were taken at the same magnification (×10). cp, compact and round pellets; hp, hyphae around pellets surface.

While the genetic alteration is important to mutant strains for hyper citric acid, the culture condition plays an important role, too. Through genetic alteration, strains are likely to be relaxed in regulation and capable of devoting their metabolic machinery to producing key biosynthetic enzymes, resulting in over-production of metabolites to the level needed for economical industrial use [11]. In present study, we paid more attention to screening mutant strains and carrying out fermentation experiments. Whether key genes may change between H4002 strain and 4# strain will be studied in our future experiments.

At present, several mutagenesis methods are employed to improve microbial strains. Every procedure has distinct advantage. Depending on uniquely physical and chemical properties, heavy ion mutagenesis methods has been used to improve the production ability of microbial and proved to be an efficient method for microbial breeding.

## IV. CONCLUSION

The study shows that $^{12}C^{6+}$ beam irradiation can cause obvious changes in the fermentation characters and pellets morphology of *Aspergillus niger*. It is ascertained that these changes lead to an increase in the content of citric acid by H4002 strain and also suggest that mutant H4002 possesses enhanced ability for sugar katabolism and citric acid production. It is proved that heavy ion irradiation is an efficient method for breeding hyper citric acid strain.

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